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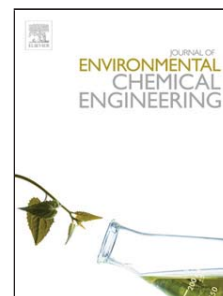
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# Toxicity assessment of four pharmaceuticals in aquatic environment before and after ferrate (VI) treatment

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## Highlights

- Ferrate(VI) at pH 6 with 3 mg/L is effective to remove the studied pharmaceuticals.
- Toxicity was assessed using Bio-fix luminescent test and zebrafish animal model.
- Simvastatin at 10µg/L exhibited high toxicity in comparison with other pharmaceuticals.
- Ferrate(VI) treatment significantly reduced the toxicity of simvastatin
- Ferrate(VI) itself did not produce additional toxicity after dosing into the test solutions

## Abstract

Micro-pollutants in aquatic environment are an emerging challenge to the human health and ecosystems. This study was to investigate the acute toxicity before and after ferrate(VI) treatment for four pharmaceuticals (simvastatin, ivermectin, fluoxetine and oxytetracycline) at concentrations of 10 and 100 µg/L, respectively. Zebrafish animal model and *Vibrio fischeri* luminescent test were employed to achieve the study targets. It is the first effort using

the stated methods to assess toxicity of the selected pharmaceuticals before and after ferrate(VI) treatment when biochemical responses of catalase (CAT), tumour necrosis factor alpha (TNF- $\alpha$ ), interleukin 1 beta (IL-1 $\beta$ ) and B-cell lymphoma 2 (Bcl-2) were assessed in the zebrafish model. The results firstly revealed a significant change in the gene expression of CAT ( $p < 0.001$ ), TNF- $\alpha$  SOD 1 ( $p < 0.01$ ), and Bcl-2 ( $p < 0.05$ ) for simvastatin at low concentrations, which exhibited high toxicity in comparison with other pharmaceuticals. Ferrate(VI) treatment significantly reduced the toxicity of simvastatin by partially removing it during the treatment process and ferrate(VI) itself did not produce additional toxicity in the effluent.

## Keywords

Ferrate(VI), pharmaceuticals, toxicity assessment, waste water treatment, zebrafish

## 1. Introduction

Pharmaceuticals and personal care products (PPCPs) are used for health or cosmetic reasons, including therapeutic and veterinary drugs, fragrances, and cosmetics [1]. PPCPs and endocrine disrupting chemicals are categorized as micro-pollutants, which have been detected and persist in water bodies throughout the world [2]. The impact of micro-pollutants on environment is always controversial. The recent identification of new compounds which can cause an adverse effect on aquatic animals has raised serious concerns in the scientific community. The removal of micro-pollutants has become quite challenging to the wastewater industries as they are present in water at a very low concentration ranging from several ng/L to  $\mu\text{g/L}$ . Ozonation, nanofiltration, activated carbon absorption and reverse osmosis are widely executed in process of removal of micro-pollutants from waste waters. Though these technologies exhibited a significant performance in the removal of micro-pollutants they are not 100% efficient in most of the cases [3]. In search of more efficient technologies, one of the efforts is to develop and use the ferrate(VI) technology. Ferrate(VI) exhibits dual roles as oxidation and coagulation in water and waste water treatment [4] and recent studies showed that ferrate(VI) outperforms to other oxidants in wastewater treatment process [5-10].

Pharmaceutical chemicals are referred as drugs which can be a single or combination of substances and are administered to restore, correct or modify physiological functions by an immunological, pharmacological, or metabolic action [11, 12]. Simvastatin (Sim) is a member of statin's group of hypolipidemic drugs, which is most commonly prescribed drug in the treatment of coronary heart diseases Simvastatin is found in low concentrations in the environment but only limited data are available in regards to toxicity [3].

Ivermectin (Ive) is one of the most commonly used anti-parasitic agents in domestic and agricultural activities. The Ivermectin passes into the environment in the unmetabolized form as faeces, which is harmful and poses a high risk for the environment [13].

Fluoxetine (Flu) is a member of the serotonin reuptake inhibitors (SSRI) class of antidepressants. It is widely spread in many countries and a study show presence of fluoxetine in effluents of 162 wastewater treatment works. Fluoxetine is found in several ng/l in water bodies and only limited data on toxicity is available [14].

Oxytetracycline (Oxy) is one of the widely used antibiotics to treat or prevent diseases in human and veterinary medicine. Oxytetracycline can reach environment through faeces of treated animals or feed itself. Certain studies showed that high concentration of antibiotics can destroy aquatic organisms such as fish, invertebrates and algae [15]. Properties of the pharmaceuticals for this study are shown in Supplemental Material, Table S1.

Zebrafish (*Danio rerio*) is a tropical fish belonging to the family Cyprinidae. It is widely used and an attractive model in toxicity testing because of its unique features such as easy maintenance of fish facility in labs, production of eggs in large number, transparent eggs for easy observation, rapid growth of embryos to adult fish, very sensitive to chemicals, and the genome is fully sequenced [2]. Recent studies reported that the exposure of pharmaceuticals caused a change in expression of certain genes such as cholinesterase (ChE), catalase (CAT), lactate dehydrogenase (LDH), vitellogenin (VTG), serotonin transport protein (SERT), 5-hydroxytryptamine 1A (5HT1A), and glutathione S-transferase (GST) activity [13, 16].

Although a number of researches have paid attention to using ferrate(VI) to treat various emerging micro-pollutants and some work reported also to assess the relevant toxicity of the treated effluents, a little of the work has been known to assess the eco-toxicity of ferrate(VI) treated water containing the stated four pharmaceuticals (Sim, Ive, Flu and Oxy)

[6]. In this study, the performance of ferrate(VI) to remove the four pharmaceuticals with low concentrations was evaluated. Moreover, the toxicity of the effluent consisting of the stated drugs before and after ferrate (VI) treatment was assessed using zebrafish animal model and biofix luminescent bacterial test.

## 2. Materials and methods

### 2.1. Test chemicals

The studied pharmaceuticals (simvastatin, ivermectin, fluoxetine and oxytetracycline) were purchased from Sigma Aldrich, which all comply with the European Pharmacopoeia Reference Standard. Other chemicals, methanol ( $\geq 99.9\%$ ), potassium ferrate(VI) ( $> 90\%$ ), acetonitrile (99.8%), fluorescein dye (95%), acridine orange dye (75%) and qPCR kit were purchased from Sigma Aldrich. Solid-phase extraction (SPE) cartridge (200mg / 6mL, 1g / 20mL) was purchased from Phenomenex, and deionised water was generated from B114 deionizer. .

### 2.2. Solid-phase extraction (SPE) and UV/Vis spectroscopy measurement

Stock solution (10 mg/L) of test chemicals was prepared using deionised reagent water. The model wastewater samples (10 and 100  $\mu\text{g/L}$ , respectively) were prepared by diluting stock solution with tap water for the experiments. The quality of tap water was measured routinely during the test period which includes averaged pH of 6.9; DOC of 0.112 mg/L; and turbidity of 0.2 NTU. The properties of tap water in Scotland are also available in Scottish Water annual water quality report

(<https://www.scottishwater.co.uk/assets/domestic/files/you%20and%20your%20home/water%20quality/annualwaterqualityreport2009.pdf>)

Samples were extracted using Phenomenex strata-X reversed phase cartridges. Cartridges were preconditioned and equilibrated. After loading sample it was washed, dried and then eluted using specific solvents as shown in Table 1. Final elute was 5 ml in volume and the flow rate of 5-10 ml per minute was maintained for the whole extraction process [5, 17].

UV/Vis spectroscopy measurement is one of common methods that researchers to select to analyze concentrations pharmaceuticals [18 – 20]. This method was adapted in this work to analyze concentrations of the studied pharmaceuticals (at two concentrations, 10 and 100  $\mu\text{g/L}$ , respectively). Calibration curves for test chemicals were constructed using the same solvent from elution step in extraction procedure. Spectra scan of wavelength 200 and 1100 nm was performed and strongest absorbance wavelength ( $\lambda_{\text{max}}$ ) was determined. Calibration was triplicated. By using  $\lambda_{\text{max}}$ , the absorbance of each standard sample solution was determined. Calibration curve was constructed from the obtained absorbance values

versus sample concentration. The  $\lambda_{\max}$  / calibration equation / correlation coefficient ( $R^2$ ) values were 238 nm/  $y=12.788x/0.9936$ , 240 nm/  $y=27.029x/0.9965$ , 226nm/ $Y=48.098x/0.9709$  and 364nm/  $Y=36.528x/0.9984$  for Simvastatin, Ivermectin, Fluoxetine and Oxytetracycline respectively. In a calibration equation, y is the detected absorbance and x is the concentration of the test chemical. Detailed procedures and calibrated standards can be seen in Figures S1-S3 in Supplementary Material.



### 2.3. Ferrate(VI) treatment

Ferrate(VI) concentration was determined by dissolving commercial available potassium ferrate(VI) (Sigma) in 4M sodium hydroxide and measuring the absorbance of resulting solution at 505 nm [6].

The model test solutions (10 and 100 µg/L) were prepared and ferrate(VI) dose (1-5 mg/L) was added. The pH (4-11) was adjusted using 0.05 M sodium hydroxide or 0.1M sulphuric acid and carried out by adding pre-determined volume of either acid or alkali simultaneously with the addition of ferrate(VI). Jar test was performed under following conditions: fast mixing (max rpm, 2 min) then slow mixing (40 rpm, 20 min) and sedimentation (60 min). Six-unit stirrer (Kemiraflocculator 2000, kemwater) was used for this purpose. The supernatants were collected, filtered and analysed by SPE and UV/Vis spectrophotometer. Best working dose and pH of ferrate were determined by evaluating the residual concentrations of pharmaceuticals.

### 2.4. Bio-fix luminescent bacterial test

Bio-fix luminescent bacterial test was performed using freeze dried luminescent bacteria. This toxicity test was performed by using BioFix Lum-10 (ATP). The test involved preconditioning (preparation of solution for freeze dried bacteria, preparation of freeze dried *Vibrio fischeri*, addition of dry sodium chloride (NaCl) to sample to make 2% NaCl sample solutions), incubation for 15 min and recording the results. Saline solution and potassium chromate were positive and negative controls respectively. The test was performed and analysed according to BS EN ISO 11348-3:2008 [21].

### 2.5. Zebrafish toxicity assessment

#### 2.5.1. Fish care and maintenance

Zebrafish eggs used in this study were breed and maintained at Glasgow Caledonian University (ZEBTEC, Tecniplast). The fish facility was maintained with constant flow of conditioned water ( $28 \pm 0.5^\circ\text{C}$ , pH7.5), ventilation and 14:10 light/dark cycle. The fish were fed with brine shrimp twice every day and over feeding was strictly avoided. The day before test, two females and males were placed in 1 L breeding chambers before start of dark cycle and left over night . The eggs were collected and examined under stereomicroscope at 4 hours

post fertilization (hpf) for quality. The healthy eggs were bleached with chlorine. The eggs were incubated at  $28 \pm 0.5^{\circ}\text{C}$  and used for the acute toxicity study [2].

### 2.5.2. Embryotoxicity essay

The eggs at 6hpf were divided according to the number of treatment groups (4 test chemicals 10 & 100  $\mu\text{g/L}$  with and without ferrate (2 & 3  $\text{mg/L}$ ) at pH4 & 6. Each treatment group of 20 eggs was exposed to 5 mL of test solution, which was freshly prepared and replaced every 24 hrs. This is to make sure that the real test concentration of these pharmaceuticals always remains the same (either 100  $\mu\text{g/L}$  or 10  $\mu\text{g/L}$ , respectively) within the exposure period till 120hpf. Each test group was closely monitored for toxic endpoints throughout the treatment process.

### 2.5.3. Quantitative real-time PCR (qRT-PCR)

The embryos after treatment process were homogenized with 1 ml TRIzol® Reagent and incubated for 5 min at room temperature. This step allows complete dissociation of nucleoprotein complex. 200 $\mu\text{L}$  Chloroform was added, vigorously shaken for 30 sec and allowed to stand for 3 min. All the tubes were then centrifuged at  $12000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . This resulted in phase separation. The top aqueous layer which contains RNA was separated, isopropanol (500  $\mu\text{L}$ ) was added and allowed to stand for 10 min. All the tubes were then centrifuged at  $12000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . RNA pellet formed during centrifugation was retained and remaining contents were discarded. 75% ethanol was added to RNA pellet and washed by centrifuging ( $7500 \times g$ , 10 min,  $4^{\circ}\text{C}$ ). Ethanol was evaporated by allowing it to stand at room temperature. The RNA pellet was resuspended with RNase-free water (25  $\mu\text{L}$ ) and incubated (15 minutes,  $55\text{--}60^{\circ}\text{C}$ ). The RNA concentration was measured by using Nanodrop spectrophotometer. It was then stored at  $-80^{\circ}\text{C}$  and used for cDNA synthesis.

cDNA was synthesized from total RNA by using Roche Transcriptor High Fidelity cDNA Synthesis Kit according to manufacturer's protocol. The cDNA concentration was measured using Nanodrop spectrophotometer. The Platinum® SYBR® Green qPCR Super Mix-UDG master mix was used to analyse gene expression according to manufacturers protocol.

Biorad CFX96™ Real-Time PCR detection system was used for qRT-PCR which was performed by UDG incubation at  $50^{\circ}\text{C}$  for 2 min, Taq polymerase activation at  $95^{\circ}\text{C}$  for 2 min and finally amplification (40 cycles) at  $95^{\circ}\text{C}$  for 15 sec. then  $55^{\circ}\text{C}$  for 30 sec. and  $72^{\circ}\text{C}$

for 1 min. Fluorescence signal detection was measured at 72°C. Ct based relative quantification of a candidate gene was calculated by using  $2^{-\Delta\Delta CT}$  method and normalizing to Ct of the zebrafish 18s RNA gene.

## 2.6. Statistical analysis

GraphPad Prism (version 7.0 from GraphPad software Inc. San Diego, CA, USA) was used for statistical analysis. All comparisons were performed using one way ANOVA, non-parametric test and Dennett's multiple comparison test at 95% confidence interval. Statistical significance was considered when a level of p is less than 0.05. \*p< 0.05; \*\*p< 0.01; \*\*\*p< 0.001; \*\*\*\*p<0.0001.

## 3. Results and discussion

### 3.1. Performance of ferrate (VI)

The efficiency of ferrate(VI) to remove pharmaceuticals can be seen in Figure 1. For most cases, a ferrate(VI) dose of 3 mg Fe/L achieved higher percentage removal of all four pharmaceuticals. Among them, Fluoxetine was relatively removed efficiently (70%) in comparison with Ivermectin, which was removed by 25% only for the same operating conditions compared. Comparing with two starting pharmaceutical concentrations, 10 µg/L and 100 µg/L, slightly more removals for Sim, Flu and Oxy were achieved for the higher concentration samples but removal efficiency of Ive was the lowest no matter what starting concentration was. The efficiency of ferrate(VI) to remove individual compound from the mixture of four pharmaceuticals was almost the same as that from the single compound solution. The effect of pH on the pharmaceutical removal was studied by conducting experiments using initial 100µg/L pharmaceutical concentration samples (Sim, Ive, Flu and Oxy) and ferrate(VI) doses (1 to 3 mg/L) for the pH range of 4-10, when the best percentage removal was achieved at pH6 for most pharmaceuticals studied (Figure 2).

The effect of solution pH on the removal of pharmaceuticals might be considered from ferrate(VI) speciation against solution pH. There are four ferrate(VI) species in aqueous solution that depend on pH:  $H_3FeO_4^+$ ,  $H_2FeO_4$ ,  $HFeO_4^-$ , and  $FeO_4^{2-}$ , and the corresponding dissociation constants are  $pK_1$  1.6±0.2,  $pK_2$  3.5, and  $pK_3$  7.23, respectively [22].  $FeO_4^{2-}$  is the dominant species in alkaline conditions, and  $HFeO_4^-$  predominates in mildly acidic

conditions. Ferrate(VI) has a higher oxidation potential at low pH (2.2 V) than in the alkaline condition (0.72V) [4] and thus the lower the solution pH, the stronger oxidation potential of ferrate(VI) ( $\text{HFeO}_4^-$  predominates) although the stability of ferrate(VI) decreases at low pH solutions.

The reactions of ferrate(VI) with some of pharmaceuticals have been studied in order to explore possibilities of using ferrate(VI) to remove pharmaceuticals from water and waste water [e.g., 23–25]. Pharmaceuticals studied include carbamazepine, sulfonamide antimicrobials, trimethoprim, tetracycline, ibuprofen, ciprofloxacin, enrofloxacin, and diclofenac. There are no published work for applying ferrate(VI) to degrade four pharmaceuticals chosen in this study and this research has thus explored the possibilities of this and attempted to obtain the optimal operating conditions. Future work aims to establish the stoichiometry and oxidation products of the reactions between these pharmaceuticals and ferrate(VI).

### 3.2. Assessment of toxicity of ferrate treated samples

#### 3.2.1. Bio-fix luminescent bacterial test

Bio-fix luminescent bacterial test results are shown in Figure 3. The concentration of samples was 100  $\mu\text{g/L}$  at a pH range of 4 to 11 was performed. The result shows that all the Sim samples and samples at pH4, 10 and 11 were toxic when compared to positive control (saline blank) and negative control (potassium chromate).

#### 3.2.2. Embryotoxicity test

##### 3.2.2.1. Acute toxicity testing

In this study, the test process resulted in mortality, hatching delay, abnormalities (abnormal spinal cord formation, heart and yolk sac, pericardial oedema, and abnormal eye and tail formation), and a decrease of the heart beat in zebrafish embryos and the effect is specific to the type and concentration of pharmaceuticals studied and solution pHs. Sim has shown toxic effect on zebrafish embryos even at a very low concentration of 100  $\mu\text{g/L}$  at pH6. The

mortality, heart rate and total abnormalities were significantly increased, whereas hatching was significantly decreased when compared to the control sample. After treatment with ferrate(VI) (2 and 3 mg/L), Sim still had toxicity although it was partially reduced, reflecting incomplete removal of Sim by ferrate(VI) from the test samples (Figure 4). However, simvastatin 10 µg/L showed a nonsignificant change in the mortality, hatching, heart rate and abnormalities when compared to the control (Figure 5).

Sim (alone or mixture) was the most toxic test compound for zebrafish. It has clearly induced statistically significant effects even at a very low concentration (100 µg/L). It is very lethal even at 100 µg/L and resulted in 80% mortality. The results observed from this study are clearly identical to that was previously reported; that Sim with 500 and 5000 µg/L exposure to zebrafish embryos resulted in 100% mortality at 32 hpf [3]. Furthermore, lower hatching and heart rate was recorded when compared to the control. Although all the test compounds showed abnormalities, the number and severity of abnormalities were observed high with Sim. Bio-fix luminescent bacterial test also showed the similar results, Sim is the most toxic compound observed from this study when compared to other test compounds.

Except Sim at 100 µg/L, other pharmaceuticals samples (100 µg/L) and all 10 µg/L samples exhibited no significant change in toxic end points (Figures 4 and 5). It is also clearly seen that the mixture of the four pharmaceuticals (10 µg/L each) caused some significant changes in mortality, hatching and total abnormalities (Figure 5). This may be because of the Sim in the mixture or may be by-products formed from interactions. However, long term exposure to a compound, an interaction with other compounds and the formation of by-products can't be ruled out.

The embryo toxicity study was also performed at pH4 and the results were shown in Figure 6. At pH4, all the test samples including the control sample showed significant toxicity when compared to the control sample at pH7. As pH4 control showed significant toxicity and pH 6 control did not, the pH range from 2 to 12 (without pharmaceuticals) was studied to determine the influence of pH on the toxicity (Figure 7). The result showed that the pH range from 2 to 4 and 10 to 12 were toxic and pHs5-9 was non-toxic, which is suitable for the zebrafish embryo growth. The importance and influence of pH can also be seen in Figure 3.

Although there may be a difference in the tested concentration from that in the environment, Sim showed high toxicity to both zebrafish embryos and *Vibrio fischeri* when

compared to other test compounds (Figures 4 and 5). Other three compounds, Ive, Oxy and Flu, at 10 µg/L, haven't shown any toxic impact on zebrafish embryos (Figures 4 and 5). On the other hand, high concentration of Ive (100 µg/L) showed a significant increase in abnormalities ( $p < 0.05$ ) when compared to the control sample and samples of Oxy ( $p > 0.05$ ) and Flu ( $p > 0.05$ ) (Figure 4).

### 3.2.2.2. Expression changes of anti-oxidant and inflammation genes

Different environmental factors, including increased temperature and oxygen, salinity, transition metal ions, pesticides and pollutants, can induce oxidative stress in aquatic animals [26]. Chemicals, including pharmaceuticals, have been reported to cause oxidative stress in farm fish [27 – 29]. Sim (10 and 100 µg/L) caused significant changes in the expression of antioxidant gene (CAT), inflammation related gene (TNF- $\alpha$ ) and apoptosis related gene (Bcl-2). Ive (10 and 100 µg/L) showed significant change in CAT gene expression, whereas Flu, Oxy and ferrate(VI) by themselves showed no significant changes in expression of test genes. The mixture of four compounds (both 100 and 10 µg/L) showed significant effects on the expression of test genes (Figures 9 and 10).

In this study, the expression of CAT gene was up-regulated significantly by Sim (10 and 100 µg/L) after a 5-day exposure (Figures 8 and 9). Change in the expression of CAT gene was reported when zebrafish were exposed to atrazine, silver, copper, cadmium and zinc nanoparticles [27]. It is interesting to see that mixture of these compounds changed the expression of the entire test genes, showing significant toxicity when compared to the control sample and individual compounds (Figures 9 and 10).

The identification of a large number of oxidative stress related genes in zebrafish has made the path easy for the researchers to study the impact of chemical pollutants and biological toxicity. For this reason, we have selected and examined the representative genes (SOD, CAT and GPX). This study helps to determine whether the selected genes have the ability to serve as bio molecular endpoints for pharmaceutical exposure in zebrafish [30].



The fish can fight against the elevated reactive oxygen species (ROS) levels in their body with SOD, CAT and GPX. They convert superoxide anions ( $O_2^-$ ) into hydrogen peroxide ( $H_2O_2$ ) and which is further converted to water ( $H_2O$ ) and oxygen ( $O_2$ ). Any change in the expression of these antioxidant enzymes may result in the excess  $H_2O_2$  and other oxy-radicals in the body [31]. It is also reported that the cells are capable of dealing with increased ROS levels up to a certain extent without any adverse effect on cellular viability or function. This might be the possible reason for the change in ROS-related gene expression to reduce the ROS levels in cells (compensatory mechanisms) and no toxicity (metabolic or cellular dysfunction) [30].

Figures 9 and 10 clearly show that the expression of Bcl-2 was inhibited by Sim and mixture solutions (10 and 100  $\mu\text{g/L}$ , respectively). Similar results were reported after atrazine exposure [30]. Bcl-2 moves to the site and eradicate generated free radicals and this further reduces oxidative stress and prevents apoptosis. Liu et al. [32] and Deng et al. [33] reported inhibition of Bcl-2 mRNA expression in zebrafish by oxidative stress [30].

Flu (10  $\mu\text{g/L}$ ) has shown the change in the expression of SOD gene when compared to the control. The lower concentration might have stimulated the gene expression and might get reduced with the increase in the concentration of pharmaceutical. Similar results were reported by Jin et al. [30], where 10 and 100  $\mu\text{g/L}$  of atrazine increased SOD, CAT and GPX expression, whereas 1000  $\mu\text{g/L}$  did not show any change in the same genes [27].

IL-1 $\beta$  and TNF- $\alpha$  (proinflammatory cytokines) belongs to the cytokine family, they migrate to the sites of injury or infection as a response to the stimuli. The expression of these genes in the samples represents injury or infection. Sim (100  $\mu\text{g/L}$ ) and mixture (10 and 100  $\mu\text{g/L}$ ) showed a significant change in the expression of TNF- $\alpha$  gene (Figs. 9 and 10). The mixture (100  $\mu\text{g/L}$ ) has shown its influence on IL-1 $\beta$  gene expression. This may be because of the higher concentration of pharmaceuticals (100  $\mu\text{g/L}$  each and 400  $\mu\text{g/L}$  in total) in the medium or formation of by-products [34]

In Fig. 10, only Sim10 (Sim, 10  $\mu\text{g/L}$ ) and mix 10 (mixture solution of four compounds at concentration of 10  $\mu\text{g/L}$  each) have shown changes in Bcl-2 gene expression. The presence of Sim in the mixture solution and interactions among four compounds, resulting in potential by-products, could be possible causes.

### 3.2.2.3. Acridine Orange staining

Apoptosis (cell death) was studied by using Acridine Orange staining. The bright spots in Figure 11 represent damage of the cells or body parts. The intensity of bright spots is directly proportional to the cellular damage hence toxicity. Staining showed bright spots in Sim and Ive group samples suggesting that these pharmaceuticals were toxic after 5-day fertilisation (Figure 11).

In multicellular organisms, the cell count is very tightly regulated by controlling cell division and programmed cell death which is commonly called as apoptosis. Acridine orange can permeate apoptotic cells and binds to DNA where normal healthy cells are non-permeable to acridine orange. The bright spots indicate the bond between acridine orange and cell DNA, a feature of apoptosis. From Figure 11, it is clearly seen that all the pharmaceutical samples in the study have caused apoptosis but the intensity of the cell damage varied. The intensity of apoptosis is directly proportional to bright spots in staining. It is interesting to see staining, particularly in the heart region of zebra fish in Sim and Ive cases. Certain studies reported that the myocyte apoptosis at a very low level can also cause dilated cardiomyopathy. A study reported that the accumulation of toxic test substance in the heart region of fish caused apoptosis. A study reported that the exposure to chemicals like hexabromocyclododecane (HBCD) and *microcystins* (MCs) on Zebrafish caused apoptosis in the heart area and retarded body growth. This suggests that the heart is a primary target for the pharmaceutical toxicity in zebrafish and this may lead to decrease in heart rate or failure in blood circulation and further slowing down the development of zebrafish [33, 35 – 37].

### 3.2.2.4. Fluorescein staining

Sim (100 µg/L) and Ive (100 µg/L) showed increase in fluorescein signal inside the embryo after three days fertilisation. The staining signal was significant in case of Sim (100 µg/L) when compared to the control embryos in E3 medium. This is of evidence that Sim 100 µg/L is toxic enough to damage the embryo chorion membrane, helping fluorescein to pass through it (Figure 12).



The zebrafish has been shown as an important animal model in understanding of the chemical toxicity, human disease mechanisms and drug discovery. Coupled with *in vivo* fluorescent imaging agents, it is widely used for the visualization of specific tissues [38]. As fluorescein has very limited water solubility [39] and was not absorbed into the zebrafish from the medium, it was employed to visualize the drug uptake and accumulation onto the eggs of the zebrafish in this study.

A study reported that zebrafish can absorb chemicals more efficiently from the surrounding medium into its body when the log P (octanol/water partition coefficient) of the chemical is more than 1 [35]. The fluorescence signal and absorption of the pharmaceuticals in the zebrafish may be correlated with their Log P values as a significant amount of Sim (log P = 4.51) and Ive (log P = 5.83) were absorbed (Figure 11). Moreover, the distribution of fluorescein in the egg varied with pharmaceutical used; Fluorescein itself or its mixture with Flu and Oxy did not show significant uptake into the chorion when dissolved in the E3 medium.

#### 4. Conclusions

For the study conditions reported in this paper, four pharmaceuticals (Sim, Ive, Flu and Oxy at low concentrations, 10 and 100 µg/L, respectively) can be removed up to 70% when dosing ferrate(VI) at 3 mg/L as Fe and controlling sample pH at 6. Increasing pH will decrease the removal efficiency due to less active ferrate(VI) species were present at high pH. Slightly more Sim, Flu and Oxy were removed in the higher concentration samples (100 µg/L) but removal efficiency of Ive was the lowest no matter what starting concentration was.

Toxicity was assessed based on the inhibition of light emission by test samples in the *Vibrio fischeri* Luminescent test, and developmental changes in the mortality, behaviour, and biochemical responses of CAT, TNF-α, IL-1β and Bcl-2 in the zebrafish model. Chemical permeability of zebrafish embryo chorion and apoptosis were determined by Fluorescein dye staining and Acridine orange staining respectively. Sim exhibited high toxicity in comparison with other pharmaceuticals. At pH6, Sim100 and Mix 100 showed the toxicity in all studies. And also, Sim100 displayed changes in gene expression in all cases except SOD and IL-1; Ive100 changed gene expression of CAT; Sim10 showed changes in the expression of CAT,

SOD, and Bcl-2; and finally, Mix10 has changed the expression of CAT, TNF, Bcl-2 genes. Finally, ferrate(VI) treatment significantly reduced the toxicity of Sim by partially removing it during the treatment process and ferrate(VI) itself did not show to produce additional toxicity in the effluent.

**Conflict of Interest:**

We confirm that no financial/personal interest conflicts exist when publishing this paper in the Journal of Environmental Chemical Engineering

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Figures:

Fig. 1. Performance of ferrate(VI) to remove four pharmaceuticals at pH6 (sim = Simvastatin 10 or 100 µg/L; Ive = Ivermectin 10 or 100 µg/L; Flu = Fluoxetine 10 or 100 µg/L; Oxy10 = Oxytetracycline 10 or 100 µg/L; XX mixed = individual compound in a mixture solution containing 4 compounds with concentration of 10 or 100 µg/L)

Fig. 2. Influence of pH on the performance of ferrate to remove 100 µg/L of Simvastatin (Sim), Ivermectin (Ive), Fluoxetine (Flu) and Oxytetracycline (Oxy)

Fig. 3. Influence of pharmaceuticals on bacterial bioluminescence with or without ferrate at pH4, 6, 10 and 11.

Fig. 4. Effect of 100µg/L test samples (Sim, simvastatin; Ive, ivermectin; Flu, fluoxetine; and Oxy, oxytetracycline) at pH6 on zebrafish embryos at 120 hpf before and after ferrate(VI) (2 and 3 mg/L) treatment. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$

Fig. 5. Effect of 10 µg/L test samples (Sim, simvastatin; Ive, ivermectin; Flu, fluoxetine; and Oxy, oxytetracycline) at pH6 on zebrafish embryos at 120 hpf before and after ferrate(VI) (2 and 3 mg/L) treatment. \* $p < 0.05$ ; \*\* $p < 0.01$

Fig. 6. Effect of 100µg/L test samples (Sim, simvastatin; Ive, ivermectin; Flu, fluoxetine; and Oxy, oxytetracycline) at pH4 on zebrafish embryos at 120 hpf before and after ferrate(VI) (2 and 3 mg/L) treatment. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$

Fig. 7. Effect of pH on zebrafish embryos mortality at 120 hpf. \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$

Fig. 8. Represents abnormalities in zebrafish development due to treatment with test samples (Simvastatin and Ivermectin). A represents control and the remaining show some kind of

abnormality in the development D, E and F shows abnormality in spinal cord formation; B and E shows abnormality in heart and yolk sac; C, E and F shows abnormality in tail formation. Control, A = Zebrafish embryo water; B and C = Ivermectin 100 µg/L treatment; D and E = Simvastatin 100 µg/L treatment; F and G = pH4 treatment; H = Fe 3 mg/L treatment and I = Dead egg.

Fig. 9. Effect of 100µg/L test samples (Sim, simvastatin; Ive, ivermectin; Flu, fluoxetine; Oxy, oxytetracycline) at pH6 on zebrafish embryos gene expression at 120 hpf before and after ferrate(VI) (2 and 3 mg/L) treatment.

Control = Zebrafish embryo water. Ctrl Fe 2/3 mg/l = ferrate dose without pharmaceuticals. ctrl pH6= zebrafish embryo water adjusted to pH6. Results are shown in error bar as the mean ± standard deviation (SD). \*\*\*\* p<0.0001; \*\*\* p<0.001; \*\*p<0.01; \*p<0.05

Fig. 10. Effect of 10 µg/L test samples (Sim, simvastatin; Ive, ivermectin; Flu, fluoxetine; Oxy, oxytetracycline) at pH6 on zebrafish embryos gene expression at 120 hpf before and after ferrate(VI) (2 and 3 mg/L) treatment.

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Fig. 11. Acridine Orange staining: Apoptosis in zebrafish due to treatment with test samples at pH 6 (Simvastatin and Ivermectin). Bright spots represent apoptosis. A represents Sim 100 µg/L treatment; B represents Ive 100 µg/L treatment; C represents Flu 100 µg/L treatment; D represents Oxy 100 µg/L treatment; E represents ferrate(VI) 3 mg/L treatment and F represents control. Control = Zebrafish embryo water.

Fig. 12. Fluorescein staining: Permeability of test samples (Sim and Ive) through zebra fish chorion after 24, 48 and 72 hours treatment. Fluorescence represents permeability. E3 medium represents control.



## Figures:

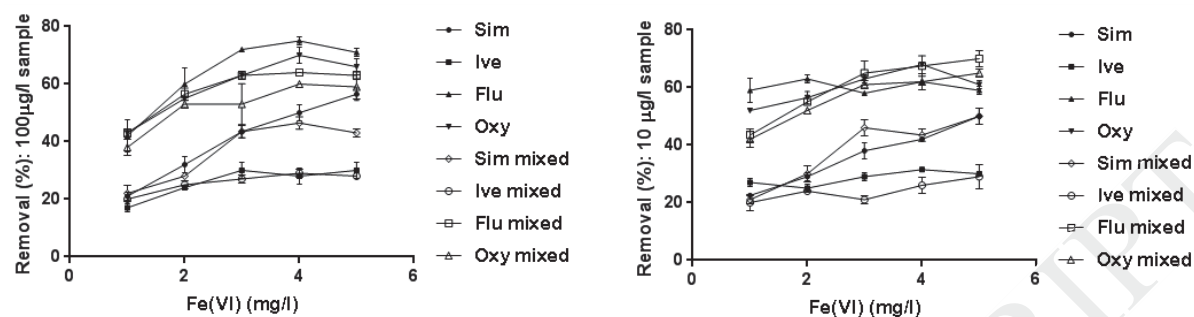


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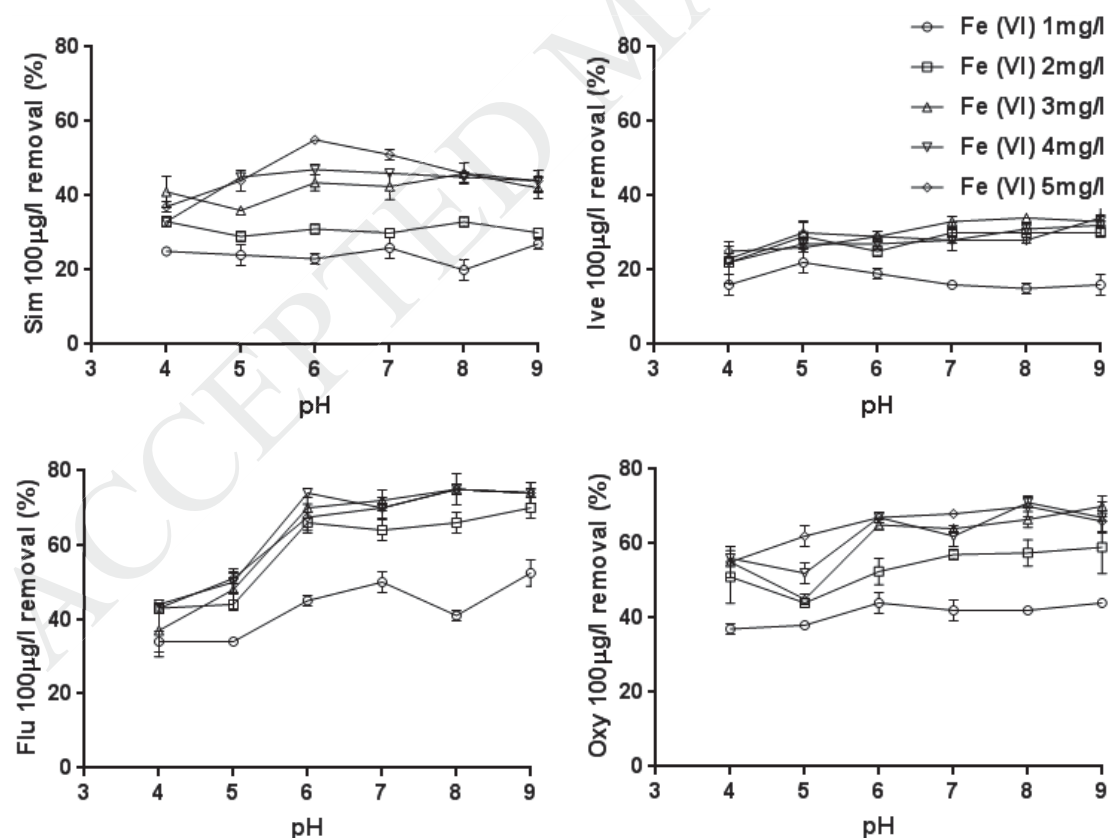


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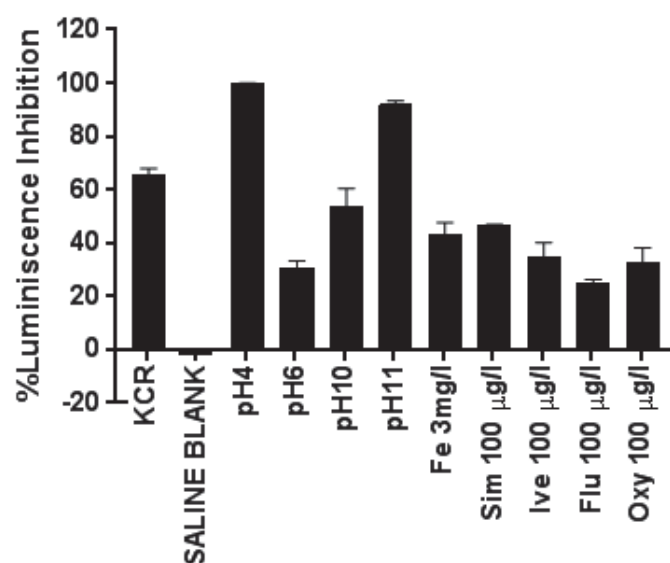


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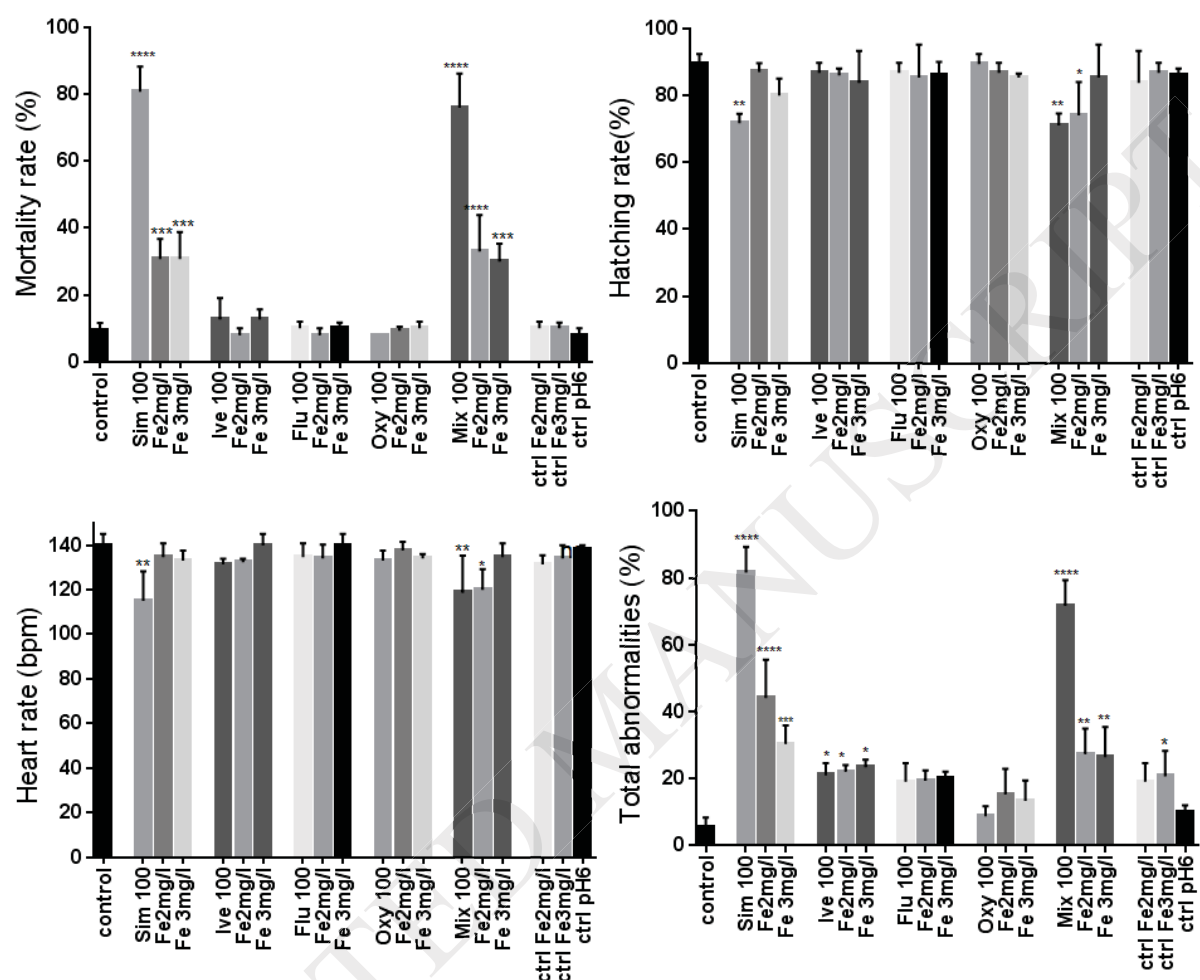


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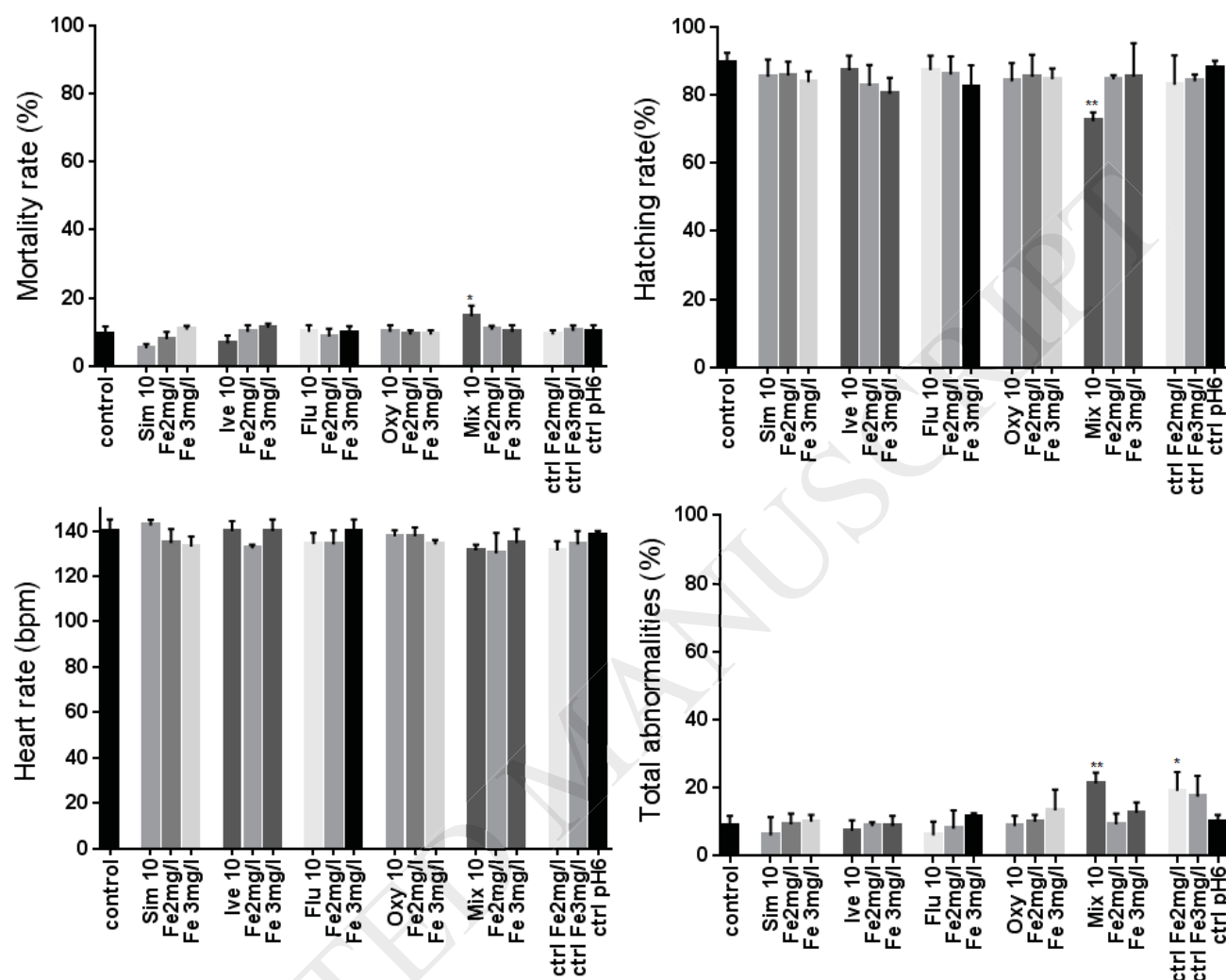


Figure 5. Effect of 10 µg/L test samples (Sim, simvastatin; Ive, ivermectin; Flu, fluoxetine; Oxy, oxytetracycline) at pH6 on zebrafish embryos at 120 hpf before and after ferrate(VI) (2 and 3 mg/L) treatment. \* $p < 0.05$ ; \*\* $p < 0.01$

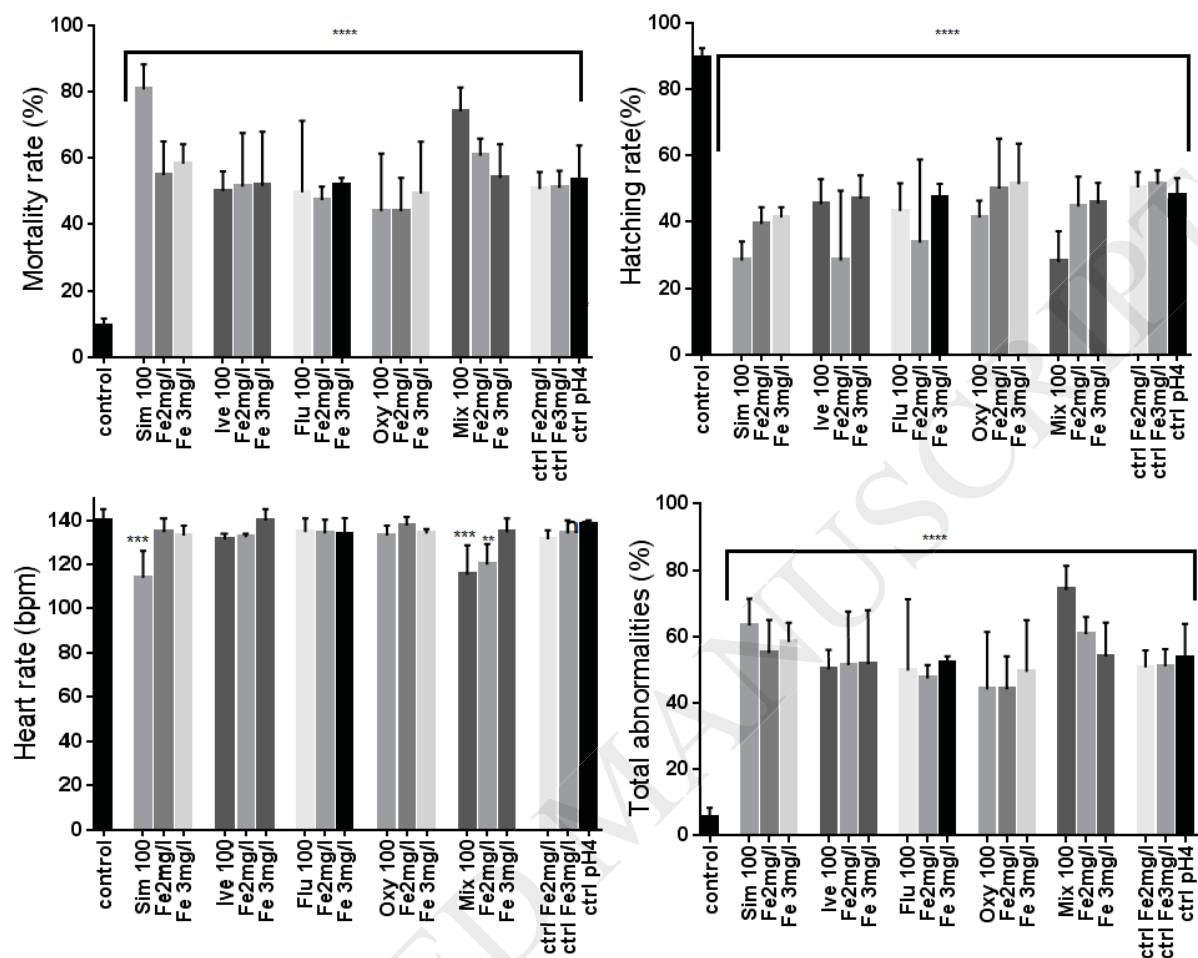


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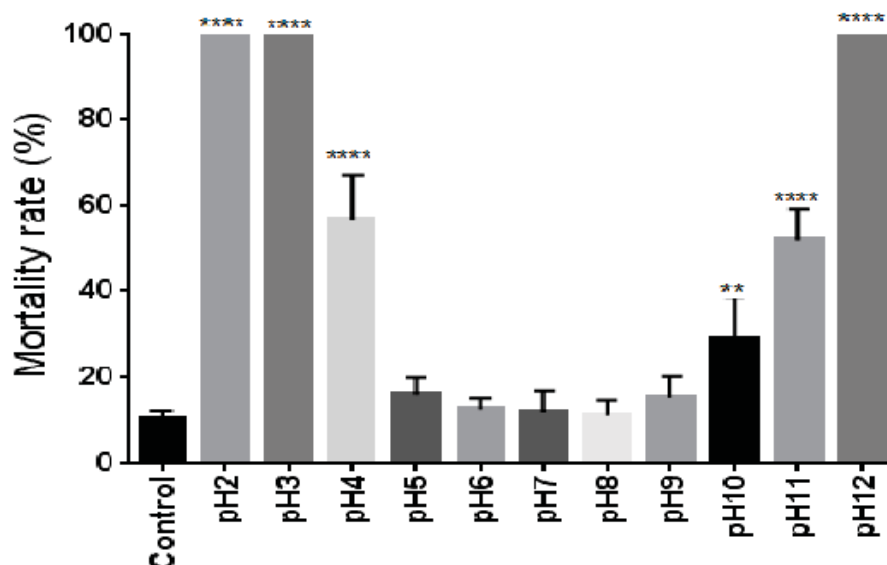


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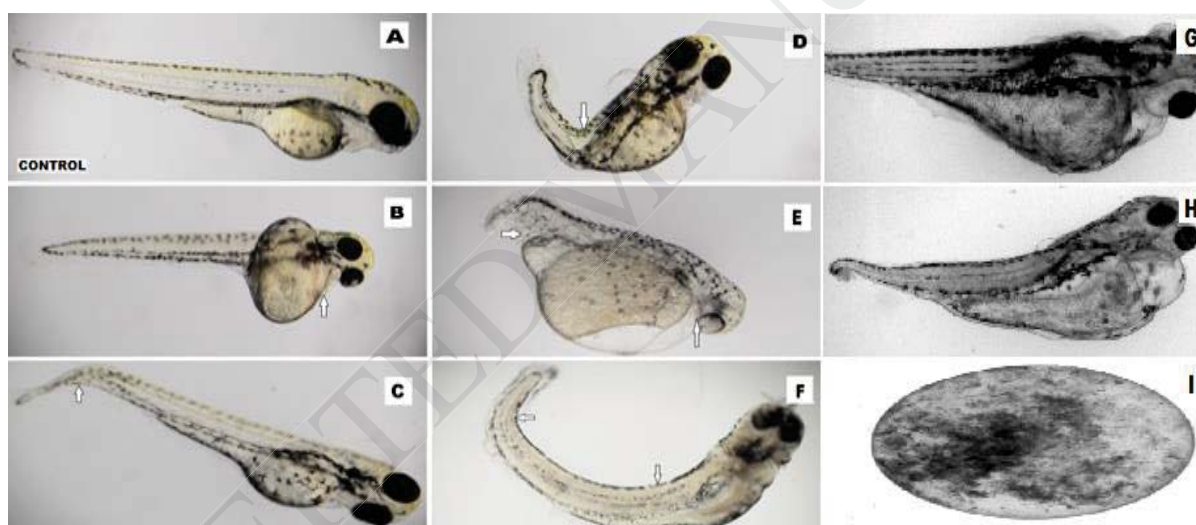


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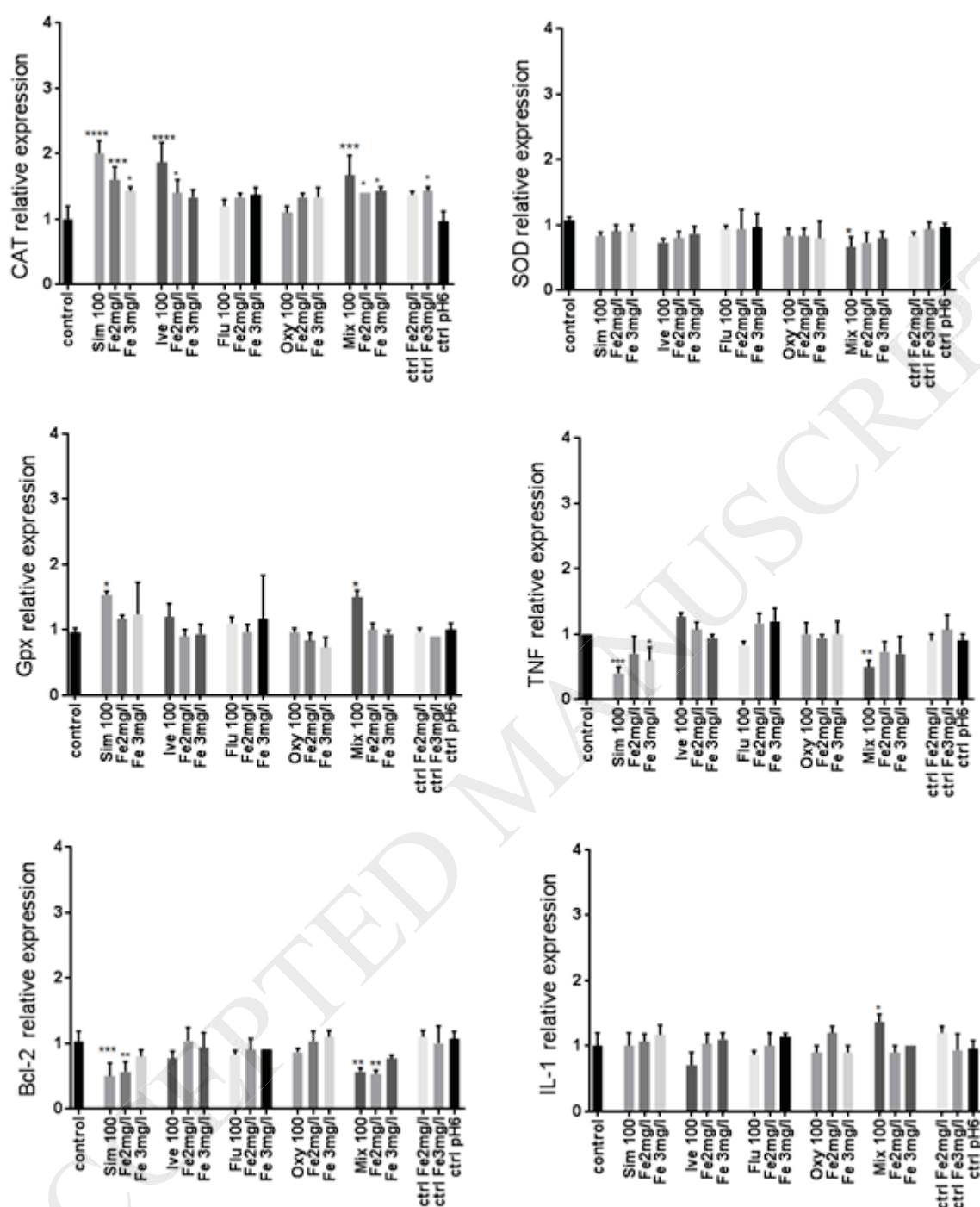


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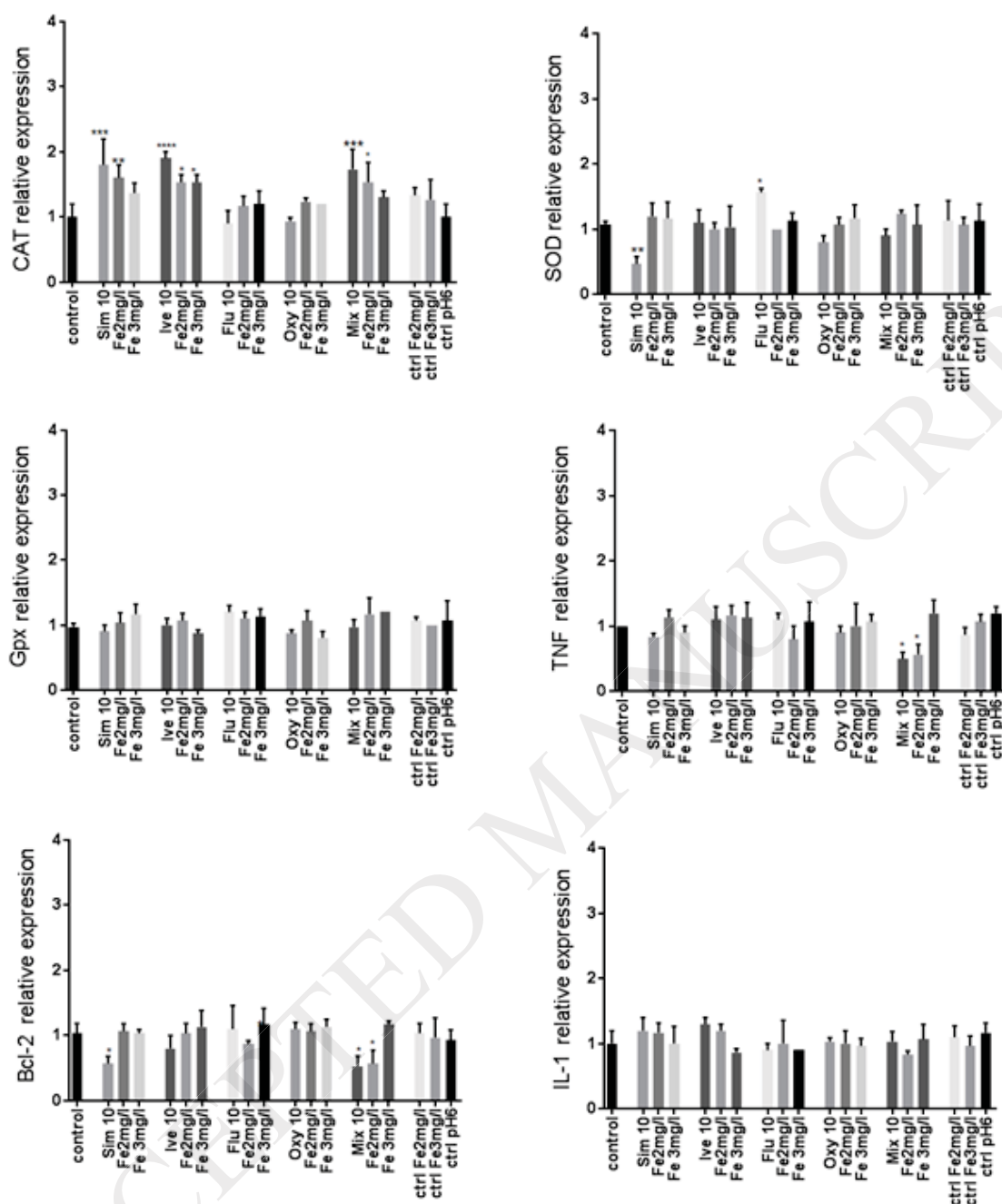


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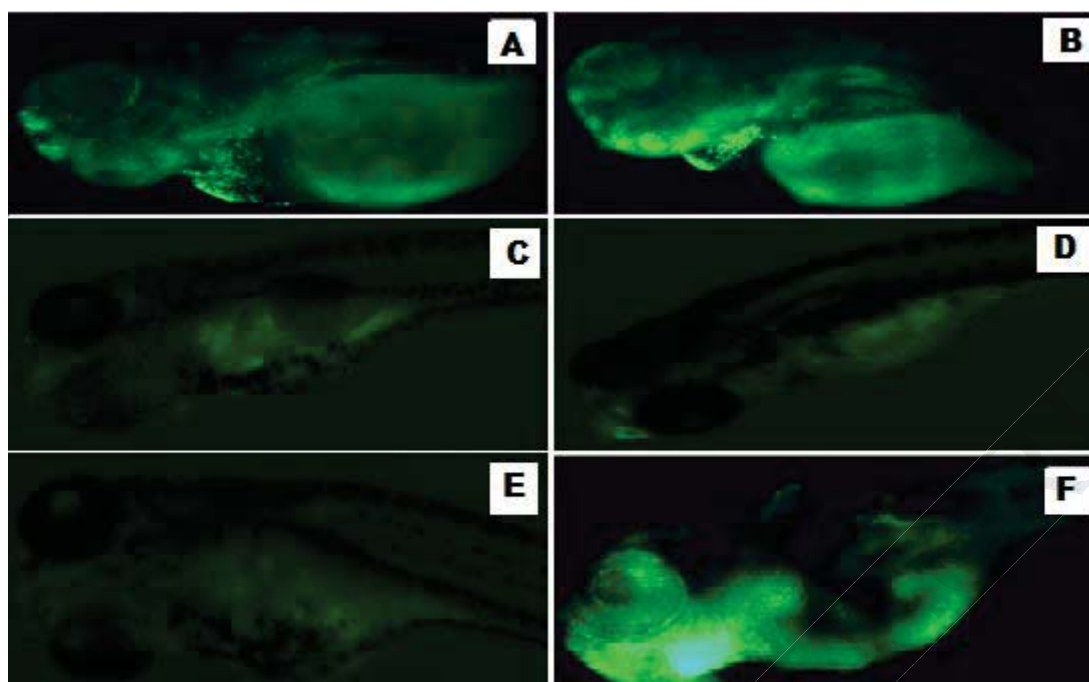


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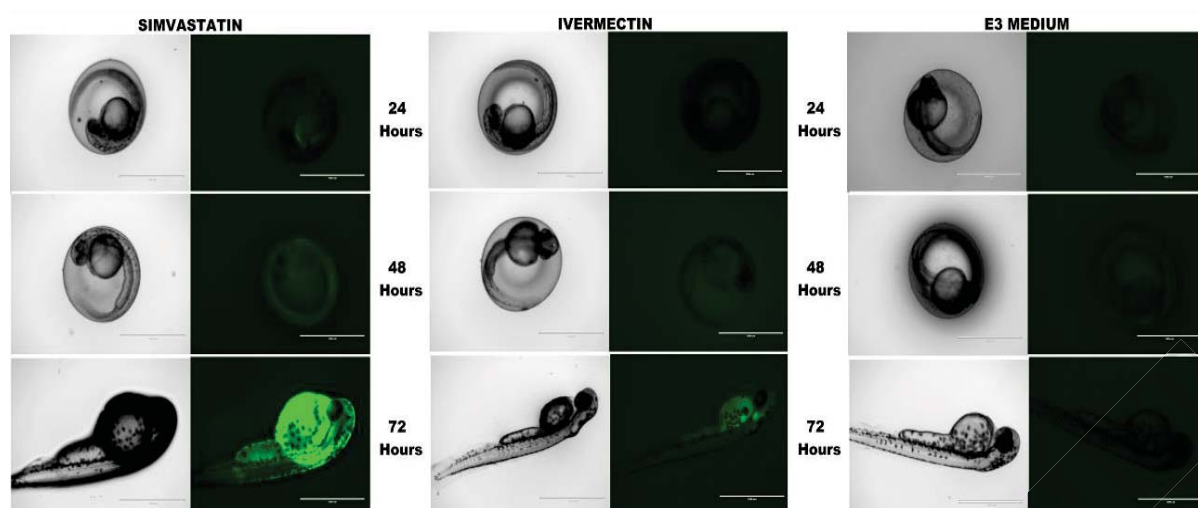


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**Tables:**

Table 1. Reversed solid phase extraction solvents and conditions

Pharmaceut ical	Pretreat ment	Precondit ion	equilibr ation	Wash 1	Wash 2	Drying	elute
Simvastatin	N/A	Acetonitr ile	Deioniz ed Water	Methanol: deionized water (35:65)	N/A	10 min  Under full vaccu m	Acetonitrile
Ivermectin	N/A	Acetonitr ile	Deioniz ed Water	Methanol: deionized water (35:65)	N/A	10 min  Under full vaccu m	Acetonitrile
Fluoxetine	Adjust pH7.8±0. 5	Acetonitr ile	Dipotass ium phospha te	Dipotassium phosphate	Methan ol	10 min  Under full vaccu m	Ammonium hydroxide:A cetonitrile (5:95)
Oxytetracyc line	Adjust pH5.8±0. 5	Methanol	100 mM Sodium acetate, pH5.8	100 mM Sodium acetate, pH5.8	Methan ol	10 min  Under full vaccu m	Ammonium hydroxide:M ethanol (5:95)